Study on the Nitrate Reductase(I)

NAD(P)H-and FMNH-dependent nitrate reductase

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It has been reported that reduction of nitrate by NAD (P) H is dependent on light, and the enzyme machinery needed for the nitrate reduction is contained in the chloroplast (3, 6, 12). These observations would explain the enhancement of nitrate reduction by light, and that the enzyme is NAD(P)H-dependent. In contrast to these reports, in 1964, Kessler(8) demonstrated that photochemically reduced NAD(P)H did not serve as an electron donor for nitrate reductase, and the nitrate reduction was not coupled to light.

In 1954, Nicholas and Nason (10) reported that $FMNH_2$($FADH_2$) also serve as electron donor for NAD(P)H-dependent nitrate reductase in Neurospora. In 1956, Stoy (14) reported that $FMNH_2$ ($FADH_2$) served as a more effective electron donor than that $NADH_2$ ($NADPH_2$) to nitrate reductase in higher plants.

The purpose of our present study is expected to determine if the nitrate reduction in higher plants is completed by a single enzyme that utilizes NAD(P)H or $FMNH_2$($FADH_2$) as electron donor, or by two different enzymes utilize two different kinds of co-factor as electron donor.

Material and methods

(1) Plant Culture Soybean seeds were soaked for two hours prior to planting in trays of vermiculate well irrigated with Haogland solution. The seedlings were grown in the green house until 10-day old. The leaves were harvested for enzyme extraction.

(2) Extraction and Purification of Enzyme In order to prepare a chlorophyll free preparation which had a minimum of gum like material, we followed the procedures of Colowick (5) for making an acetone powder. The dried acetone powder was extracted with 12 volume of cold phosphate buffer (0.2M, pH 7.5). The crude extracts were purified by ammonium sulphate precipitation and calcium phosphate gel adsorption as described by Evans and Nason (6).

(3) Preparation of Cofactors $NADPH_2$ and $NADH_2$ were chemically reduced as the method described by Lehninger (5*) $FADH_2$ and $FMNH_2$ were enzymatically reduced by the method of Felton (5*).

* The methods preparing the reduced cofactors as described by Lehninger, Felton are collected in "Methods in Enzymology" by Colowick. et al.

(4) Preparation and Assay of Nitrate Reductase Activity The assay systems were prepared as indicated in the accompanying table :

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Reaction mixture	NAD (P) H-NR*	$FADH_2$ ($FMNH_2$) -NR
KNO_3, 10^{-2}M	1 ml	1 ml
NAD (P) H, 1.0×10^{-3}M	0.25 ml	---
$FADH_2$ ($FMNH_2$) ,10×10^{-3}M	---	0.25 ml
Phosphate buffer, 0.2M pH 7.5	1.50 ml	1.50 ml
Enzyme extract	0.25 ml	0.25 ml
Final volume	3.0 ml	3.0 ml

* NR is abbreviation for Nitrate Reductase.

The reaction mixture was incubated for 30 minutes at 37°, then the reaction was stopped by adding 2 ml of Uranyl acetate reagent. This was centrifuged and the precipitate discarded. To the supernatant fraction was immediately added 1 ml of N-(naphythyl) ethylenediamine solution (0.02%) to develope the color of NO_2^-. After 15 minutes the optical density was determined with a Spectronic 20 at 540 mμ.

The optical density of the control reaction mixture containing all the constituents except the enzyme extracts was used to correct for turbidity.

(5) Definition of Enzyme Unit and Specific Activity One unit of nitrate reductase is defined as that amount of enzyme resulting in the formation of one μ mole of nitrite under this assay condition.

Specific activity is expressed as the units of enzyme per milligram of protein.

The protein in the extracts was precipitated by Trichloroacetic acid, and its amount determined by micro-Kjeldahl method.

Results and discussion

(1) Purification of Nitrate Reductase. Both NAD(P)H- and $FMNH_2$-dependent nitrate reductase to be present in the crude extract ammonium sulfate precipitate and calcium phosphate gel elute, there is a slight excess of NAD(P)-NR. Ammonium sulfate precipitation followed by gel adsorption resulted in an 12 fold purification of the enzymes under study (Table I).

Table I. Purification of Nitrate Reductase.

Fraction	Protein		Specific activity of enzyme	
	mg/ml	total amount	NAD (P) H-NR*	FMNH-NR*
Crude extract	4.49	18765	38.5	35.7
Fraction I 43% $(NH_4)_2SO_4$ ppt	2.65	588.6	66.0	54.4
Fraction II 46% $(NH_4)_2SO_4$ ppt	1.06	188.52	139.1	129.2

Fraction III Ca-phosphate gel elute	0.58	12.18	483.3	471.0

**NAD(P)H-NR=NAD(P)H-dependent nitrate reductase.

FMNH-NR=$FMNH_2$ (or $FADH_2$) -dependent nitrate reductase.

(2) Induction of Nitrate Reductase. The ten-day-old seedlings were excised at the ground level and submerged them in a medium containing desired level of substrate and inhibitor. The results of these experiments (Table II) show that: (a) appearance of the activity of NAD (P) H-NR and FMNH-NR are in identical physical properties each other, (b) synthesis of nitrate reductase is depended upon the present of nitrate, i.e. the nitrate reductase is an adaptive enzyme, (c) if the seedlings deficient in nitrate are excised and incubated in the induction medium containing nitrate, the seedlings show a linear increase in production of nitrate reductase. The longer the incubation time for seedlings, the greater amount of nitrate reductase it induced. On the contrary, if the seedlings well supplied with nitrate in the nutrient solution, the seedlings show no further increasing in production of nitrate reductase when they are in the induction medium, (d) if cycloheximide are added to the medium, the nitrate reductase can not be induced with the nitrate. The longer the incubation times for seedlings in the induction medium containing cycloheximide, the lesser amount of nitrate reductase it induced. Since the cycloheximide is an inhibitor of protein synthesis.

Table II. The Condition for Induction of Nitrate Reductase.

Induction* medium	Induction time, hour	Enzyme activity a-1 Preinduced NAD(P)H-NR	a-1 Preinduced FMNH-NR	a-2 Not preinduced NAD(P)H-NR	a-2 Not preinduced FMNH-NR
Nitrate	0	38.5	35.7	11.4	10.5
	2	38.6	35.9	19.8	15.3
	4	38.9	35.9	22.2	20.1
	16	39.6	36.3	29.5	28.2
Nitrate, cycloheximide	0	38.5	35.7	11.4	10.5
	2	32.1	31.2	7.7	7.8
	4	25.6	24.2	3.9	4.2
	16	1.12	0.77	–	–
-nitrate, -cycloheximide	0	38.5	35.7	11.4	10.5
	2	37.0	34.8	8.0	7.9
	4	33.3	32.1	6.7	6.3
	16	27.9	24.4	5.5	5.1

* The induction media was prepared as the method described by White

a-1 The seedlings were planted in a nutrient solution containing nitrate.

a-2 The seedlings were transferred to a nutrient solution lacking nitrate.

The unit expressed here is the specific activity of enzyme.

(3) Sulfhydryl requirement. In 1953, Evans and Nason (6) showed that sulfhydryl group was required for nitrate reductase activity in higher plants. This-SH group is inhibited by the p-chloromercuribenzoate (PCMB). In our experiments with a crude nitrate red-

uctase extracted from soybean leaves showed that preincubating the enzyme with 10^{-3}M or 10^{-6}M p-chloromercuribenzoate resulted in complete or 50%, respectively, inhibition of both NAD(P)H-NR and FMNH-NR. If 1 x 10^{-3}M of cysteine hydrochloride was added to the assay mixture, the inhibition due to p-chloromercuribenzoate (10^{-3}M) was reduced to 30% or mars, and little inhibition when concentration of p-chloromercuribenzoate was below 10^{-4}M (Figure I). From the date presented in the Figure 1 and Table 111, we verify that; 1. high concentration of exogenous cysteine increases in stability of NAD(P)H-and $FMNH_2$ (or $FADH_2$) -dependent nitrate reductase activity, 2. addition of cysteine prevents p-chloromercuribenzoate inhibition of enzyme activity.

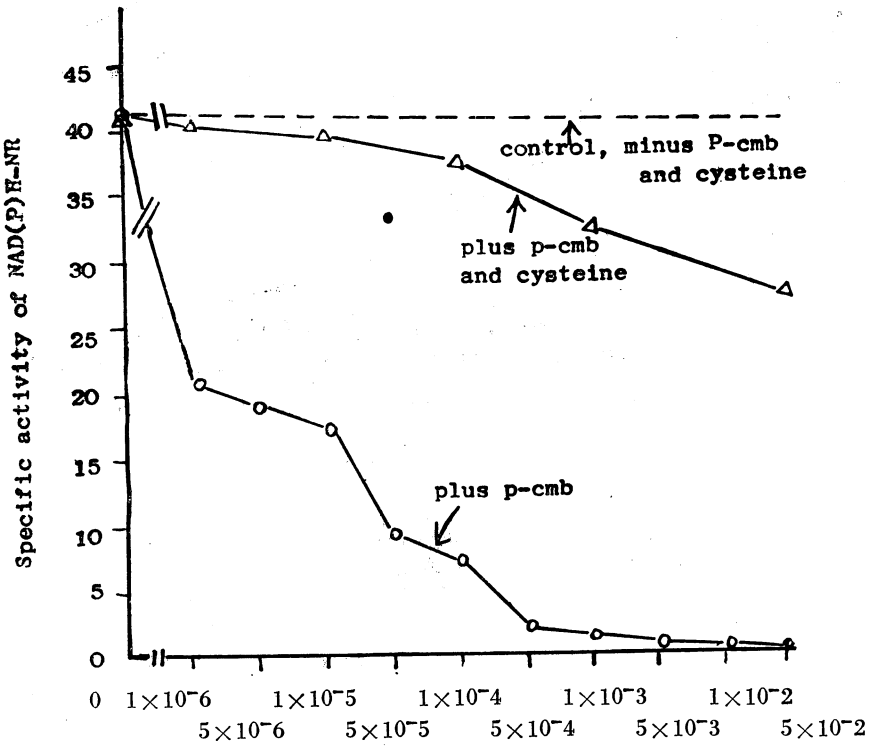
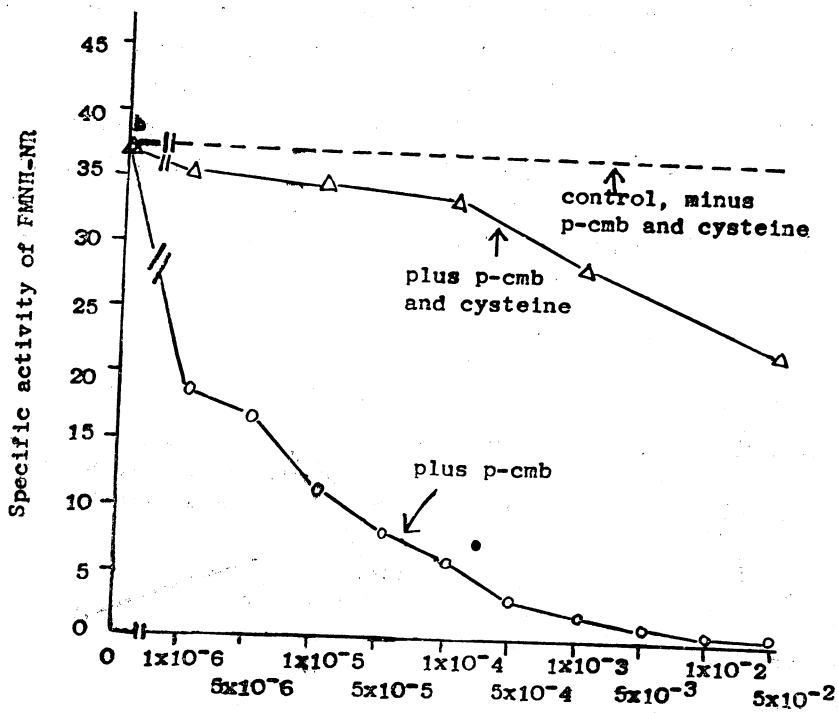


Figure 1-A, The inhibition of NAD(P)H-NR by p-chloromercuribenzoate and reversal by cysteine.



Concentratfon of p-chloromercuribenzoate (p-cmb) (M)

Figure 1-B The inhibition of FMNH-NR by p-chloromercuribenzoate and reversal by cysteine

Table Ⅲ. The Effect of Cysteine on the Extraction of Nitrate Reductase.

Reaction mixture	Specific enzyme activity	
	NAD (P) H-NR	FMNH-NR
Phosphate buffer	38.5	35.7
Phosphate buffer plus cysteine	55.1	40.0
% increase brought about by cysteine	43	20

The concentration of cyseine used here is 1.0×10^{-3}M.

(4) Thermal Stability of NAD(P)H-NR and FMNH-NR. No differential loss of NAD (P) H-NR and FMNH-NR was noted in vivo at the higher temperature. However, the data in the Table IV show that at 45°c, NAD(P)H-NR in vitro is more heat labile than the FMNH-NR. Therefore, selective denaturation by heating to 45°c could result in separation and purification of NAD (P) H-NR from FMNH-NR.

Table IV. Relative Stability of NAD(P)H-NR and FMNH-NR to Temperature

Temperature °C	Specific activity of enzyme			
	NAD(P)H-NR		FMNH-NR	
	10 min.	20 min.	10 min.	20 min.
20	28.7	29.4	27.7	28.6
30	38.5	41.0	36.1	36.6
45	23.9	18.2	33.7	32.4

From the data in The Table IV, it shows that: (a) the higher the temperature (below 45°c) for enzyme reaction, the greater enzyme activity it shows, The Q_{10} for nitrate reductase is 1.3 to 1.4 (b) the longer the incubation time for enzyme reaction, the greater amount of nitrite are produced.

(5) The Effectiveness of Electron Donors for Nitrate Reductase. For the purpose of determining the relative effectiveness of electron donors for nitrate reduction, $NADPH_2$, $NADH_2$, $FADH_2$, and $FMNH_2$ were added individually or in combination to the reaction mixture. The results (Table V) show that reduced nicotinamide adenine nucleotindes are the most effective electron donors in higher plants. $FMNH_2$ is somewhat less effective, $NADH_2$ and $FADH_2$ fall in between of them. Combination of $NADPH_2$ and $FADH_2$ gave no greater activity than did $NADPH_2$ alone. This observation are comparable to the apparent Km values of cofactor which presented in the Figure II.

Table V. Relative Effectiveness of Electron Donors for Nitrate Reductase.

Electron donor	conc.	u moles cofactor oxidized 340m/u	450m/u	u moles NO_3^- consumed	u moles NO_2^- produced
$NADPH_2$	157x10-6M	0.199	---	0.214	0.197
$NADH_2$	157x10-6M	0.185	---	0.200	0.189
$FADH_2$	262x10-5M	---	0.189	0.191	0.186
$FMNH_2$	262x10-5M	---	0.181	0.171	0.175
$NADPH_2$ plus $FADH_2$	78x10-6M 131x10-5M	0.106	0.084	0.212	0.195

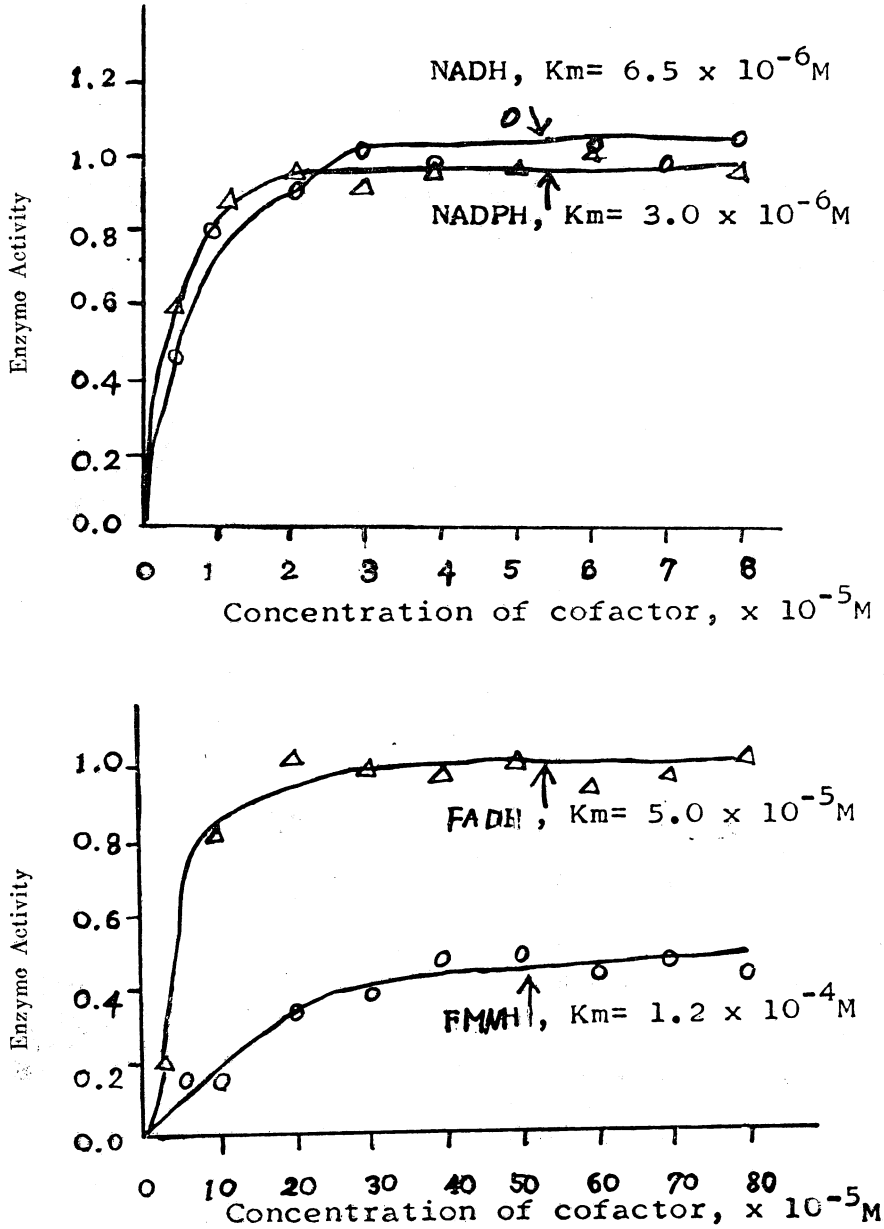
The concentration of cofactor oxidized were determined by the Extinction Coefficient: i.e. 6.24×10^6 cm^2/mole for $NADPH_2$ and $NADH_2$.

1.13×10^7 cm^2/mole for $FADH_2$ and $FMNH_2$.

Amount of cofactor oxidized, NO_3^- consumed, and NO_2^- produced were in agreement, i.e. when one mole of NO_2^- are produced, there one mole of NO_3^- are reduced, and one mole of cofactor are oxidized at the same time.

(6) Apparent Km value for the Cofactors and the Nitrate. (a). $FMNH_2$ concentration required for achieving the half maximal rate of nitrate reduction was greater than that

of $FADH_2$, and the concentration of $NADH_2$ required for half maximal rates was greater than when $NADPH_2$ was used. In general, higher concentration (10-fold to 100-fold) of reduced flavin nucleotides is required to achieve a half maximal rate of nitrate reduction than when reduced nicotinamide adenine nucleotides were used (Figure II). These results indicate that a greater affinity of the enzyme for NAD(P)H than for $FADH_2$ ($FMNH_2$), i. e. $NAD(P)H_2$ is a more efficient hydrogen donor for nitrate reductase than $FADH_2$ (or $FMNH_2$) is, (b)the concentration of NO_3^- required for one half maximal NAD(P)H-NR activity was 2.0×10^{-4} M and that for $FADH_2(FMNH_2)$-NR Was 3.9×10^{-4}M (FigIII) These results indicate that a greater affinity of the NAD(P)H-NR for nitrate than is $FADH_2$ ($FMNH_2$)-NR for nitrate.



FigII (P.57)

Figure II. The apparent Km value for NADPH, NADH, FADH, and FMNH. The velocity (EcgymeActivity)expressed here is u moles NO_2-Produced, per milligram of protein, per hour.

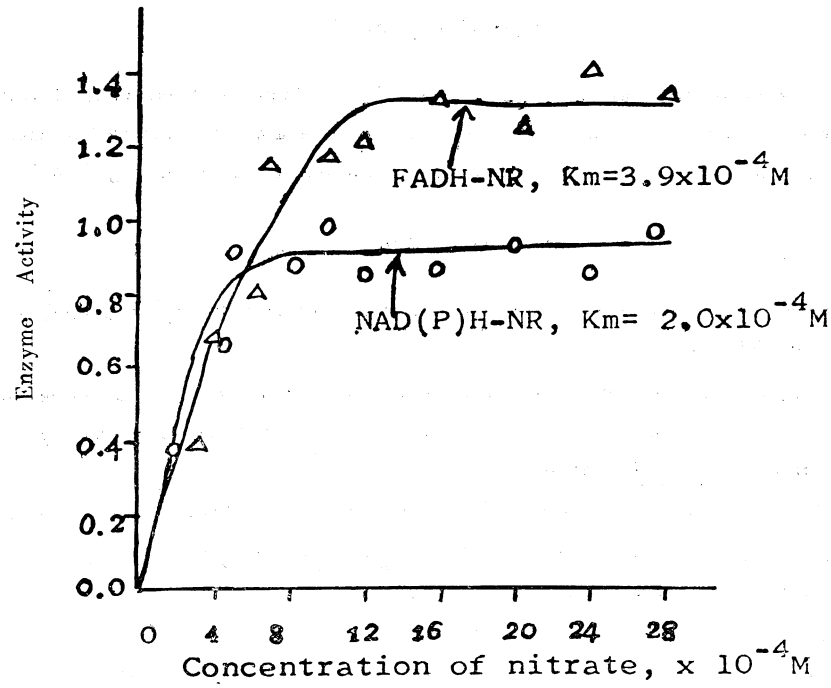


Figure III. Apparent Km value for nitrate.

Summary

1. There show a little differential activity in NAD(P)H-NR and FMNH-NR. The activity of NAD(P)H-dependent nitrate reductase predominate to that of FMNH- dependent nitrate reductase.
2. NAD(P)H-NR and FMNH-NR are equally susceptible to the inhibition of p-chloromercuribenzoate, but this inhibitory effect can be reversed by cysteine in the reaction mixture.
3. Presence of cysteine in the extraction medium results in increasing enzyme activity. NAD(P)H-NR and FMNH-NR activity are increased about 43% and 20%, compared to that of the control lacking cysteine, respectively.
4. Activity of NAD(P)H-NR decrease more rapidly that of FMNH-NR at the temperature above 45°C, i.e. NAD(P)H-NR is more heat labile.
5. Relative affinity of cofactors to enzyme are arranged, according to their apparent Km values, in order of

   $NADPH_2$> $NADH_2$ for NAD(P)H-dependent nitrate reductase.

   $FADH_2$> $FMNH_2$ for FMNH-dependent nitrate reductase.

   Relative affinity of nitrate to enzyme:

   NAD(P)H-dependent nitrate reductase> FMNH-dependent nitrate reductase.

   By comparison of the characteristics of enzyme described above, the so-called nitrate

reductase may be a mixture of NAD(P)H_and FMNH_dependent nitrate reductase. Although these kinds of enzyme are nearly identical in chemical and physical properties.

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植物體內硝酸鹽還原酵素活性之研究

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摘 要

從前一般學者都認爲於植物體內硝酸鹽還原過程中，祇有一種硝酸鹽還原酵素參與催化作用，又依酵素的特性而言，一種酵素蛋白（Apoenzyme）與一種輔酵素（Coenzyme）構成爲一種完全酵素，所以硝酸鹽還原酵素似乎不可能與兩種輔酵素（NADPH或$FADH_2$）結合。本研究爲針對此問題加以探討，究由一種酵素蛋白可利用兩種輔酵素，或兩種酵素各利用其所需的輔酵素，同時參與硝酸鹽還原作用，玆以大豆爲材料經實驗結果摘要如下：

1.在此實驗我們已獲知：當硝酸鹽被大豆吸收後，在轉變爲銨的過程中有兩種硝酸鹽還原酵素參與作用，則 NADPH和FMNH 硝酸鹽還原酵素，此兩種酵素在理化性上很近似，但是以活性而言，NADPH 硝酸鹽還原酵素大於 FMNH 硝酸鹽還原酵素。

2.NADPH 硝酸鹽還原酵素和 FMNH 硝酸鹽還原酵素的活性同樣可受 P—Chloromercuribenzoate 所抑制；可是此種抑制作用，又可被半胱氨酸（Cysteine）所克服，由此可見半胱氨酸對此兩種酵素的活性有促進作用，與對照比較其活性效率可使 NADPH 硝酸鹽還原酵素的活性約增加 43%，而對 FMNH 硝酸鹽還原酵素也可以增加約20%，所以半胱氨酸對 NADPH 硝酸鹽還原酵素之作用大於 FMNH 硝酸鹽還原酵素。

3.FMNH 硝酸鹽還原酵素較 NADPH 硝酸鹽還原酵素耐熱，當溫度大於 45°c 時 ， NADPH 硝酸鹽還原酵素之活性則急減。

4.依照Km值比較，在 NADPH 硝酸鹽還原酵素所結合的輔酵素 $NADPH_2$（$TPNH_2$）較$NADH_2$（$DPNH_2$）爲佳，而於 FMNH 硝酸鹽還原酵素則以 $FADH_2$ 比 $FMMH_2$ 爲佳。所以在大豆體內而言，參與硝酸鹽還原作用的酵素， NADPH 硝酸鹽還原酵素比FMNH硝酸鹽還原酵素爲佳。

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